FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
1038-1176 MIS:jb	09/914205

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/00190	February 24, 2000	February 24, 1999

TITLE OF INVENTION

EXPRESSING GP140 FRAGMENT OF PRIMARY HIV-1 ISOLATE

APPLICANT(S) FOR DO/EO/US

Charles D.Y. Sia; et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). - unsigned copy
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Five Assignments
Notification of the Recording of a Change
Initial Information Data Sheet

JC05 Rec'd PCT/PTO 23 AUG 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/914205	PCT/CA00/00190	1038-1176 MIS:jb

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1000.00**
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$860.00**
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$710.00**
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$690.00**
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	16 - 20 =	0	x $18.00	**$0.00**	
Independent claims	4 - 3 =	1	x $80.00	**$80.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$940.00**	
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$940.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$940.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☒				**$200.00**	
TOTAL FEES ENCLOSED =				**$1,140.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$1,140.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **192253** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Michael I. Stewart
Sim & McBurney
6th Floor, 330 University Avenue
Toronto, Ontario
Canada, M5G 1R7.

24223
PATENT_TRADEMARK OFFICE

SIGNATURE

Michael I. Stewart
NAME

24,973
REGISTRATION NUMBER

August 22, 2001
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re National Phase of International Application

Applicant	:	Charles D.Y. Sia, et al.
Appl'n. No.	:	PCT/CA00/00190
Filed	:	February 24, 2000
Title	:	EXPRESSING GP140 FRAGMENT OF PRIMARY HIV-1 ISOLATE
Docket No.	:	1038-1176 MIS:jb

August 22, 2001

BY COURIER

The Commissioner of Patents
and Trademarks,
Washington, D.C. 20231,
U.S.A.

PRELIMINARY MENDMENT

Sir:

Please amend the above-identified application as follows:

In the Specification:

Before the first line of the specification, add the following:

" REFERENCE TO RELATED APPLICATIONS

This application is a national phase application under 35 U.S.C. 371 of PCT/CA00/00190."

REMARKS/ARGUMENTS

The specification has been amended on page 1 to reflect that this application is a U.S. National Phase filing under 35 U.S.C. 371 of PCT/CA99/00766.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "**Version with markings to show changes made**."

Respectfully submitted,
SIM & McBURNEY

M.I. Stewart
Reg. No. 24,973

Toronto, Ontario, Canada,
(416) 595-1155
FAX No. (416) 595-1163

Appl. No.

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Before the first line of the specification, add the following:

" REFERENCE TO RELATED APPLICATIONS

This application is a national phase application under 35 U.S.C. 371 of PCT/CA00/00190."

TITLE OF INVENTION

EXPRESSING GP140 FRAGMENT OF PRIMARY HIV-1 ISOLATE

FIELD OF INVENTION

The present invention relates to the field of immunology, specifically HIV Vaccine Technology, and, in particular, is concerned with expressing the extracellular fragment of the envelope gene, gp140, of a primary human immunodeficiency virus type 1 (HIV-1) isolate.

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending United States Patent Application No.: 09/256,194 filed February 24, 1999.

BACKGROUND OF THE INVENTION

Acquired immunodeficiency syndrome (AIDS) is a disease which is the ultimate result of infection with human immunodeficiency virus (HIV). Currently, there is no effective vaccine which can protect the human population from HIV infection and hence the development of an efficacious HIV-vaccine and protocol for administering the same is urgently required. Previously, HIV-1 particles exhaustively inactivated by chemical treatments, a vaccinia vector encoding the whole envelope gene (gp140) of HIV-1, and purified recombinant gp120 have been evaluated as candidate HIV vaccines. Although inactivated HIV-1 virus preparations elicited a T-cell-mediated Delayed-Type Hypersensitivity (DTH) reaction in humans, and vaccinia/gp160 and gp120 recombinant vaccine candidates induced virus neutralizing antibodies, none of these immunogens have been shown to be efficacious human HIV vaccines (ref. 1, throughout this specification, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately following the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure). The inventors' interest in HIV vaccinology is to develop immunogenic and cost-effective HIV-1 DNA vaccines and consider that

their use alone or in conjunction with other forms of HIV-1 vaccine candidates will lead to the elicitation of more effective immune responses against HIV-1.

There has previously been described in granted European Patent No. 470,980 and U.S. Patent No. 5,639,854, assigned to the assignee hereof, the disclosures of which are incorporated herein by reference, *inter alia*, the identification and characterization of a T-cell epitope of the core protein, p24E, of HIV-1. There has further been described in granted U.S. Patents Nos. 5,759,769 and 5,795,955, assigned to the assignee hereof, and disclosures of which are incorporated by reference, the use of the T-cell epitope in the construction of immunogenic synthetic HIV-1 chimeric peptides comprising p24E linked to amino acid sequences of different B-cell epitopes of an envelope or core protein of HIV-1.

SUMMARY OF THE INVENTION

The present effort has turned to design and construction of HIV DNA-based immunogens capable of eliciting cell-mediated immunity (CMI). In this context, the inventors have focused interest on the extracellular envelope fragment, gp140, expressed in a primary HIV-1 isolate, HIV-1 (BX08), for the reason that this protein is rich in motifs restricted to both the murine and human Major Histocompatibility Complex (MHC) class 1 alleles.

Immunization with an appropriately constructed immunogen expressing the gp140 protein leads to the generation of peptides with class 1 binding capability to allow the induction of HIV-1-specific CTLs capable of killing virus infected cells to limit infection.

The invention described by the inventors is that they have found a plasmid designated, pCMV.gp140.BX08, expressing the gp140 gene under the control of a CMV promotor was immunogenic in BALB/c mice in the elicitation of CTL response directed against multiple epitopes of the gp140 protein that are restricted to different H-2^d class 1 gene products. It was also found that plasmids based on Semliki Forest Virus (SFV) vectors, namely pMP83, pMP84 and pMP88, also requiring the gp140 gene under the control of a CMV promoter were similarly immunogenic.

Accordingly, in one aspect of the present invention, there is provided a vector, comprising a gene encoding the extracellular fragments of gp140 of a primary HIV-1 isolate, preferably BX08, under the control of a promotor for expression of the gene product in a host organism, thereby eliciting a cytotoxic T-cell response.

The promotor preferably is the cytomegalovirus promotor. The vector may preferably be a plasmid vector having the identifying characteristics of plasmid pCMV.gp140.BX08, as shown in Figure 1. The vector also may preferably be a plasmid vector having the identifying characteristics of plasmids pMP88, pMP84 or pMP83.

The invention further includes an immunogenic composition containing the vector as well as a method of generating a cytotoxic T-cell response to HIV-1 in a host by administering to the host the immunogenic composition provided herein. Such immunogenic composition may be formulated for intramuscular immunization with a suitable carrier or may be formulated for gene gun delivery with gold particles.

The invention extends to the vector when used as an immunogen for generating a cytotoxic T-cell response to HIV-1 in a host and to the use of the vector in the manufacture of an immunogen for the generation of a cytotoxic T-cell response to the HIV-1 in a host.

The invention further includes certain novel peptides and nucleic acid molecules as set forth below.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows details of the elements of plasmid pCMV.gp140.BX08;

Figure 2 shows the nucleotide (SEQ ID No: 1) and deduced amino acid sequence (SEQ ID No: 2) of the gp140 open reading frame of the plasmid pCMV.gp140.BX08;

Figures 3A, 3B and 3C show the effector responses elicited by intramuscular injection of the plasmid, pCMV.gp140.BX08, in BALB/c mice;

Figures 4A, 4B and 4C show the effector responses elicited by gene gun delivery of the plasmid, pCMV.gp140.BX08, in BALB/c mice; and

Figure 5 is a nucleotide sequence of a BamHI fragment (SEQ ID No: 15) containing the HIV-1, gp140 nucleotide sequence for incorporation into a BamHI site of alphavirus expression vectors.

DETAILED DESCRIPTION OF THE INVENTION

A DNA immunogen is constructed using recombinant DNA technology to molecularly clone a gene of interest into a plasmid expression vector. A unique feature involving vaccination with a DNA-based immunogen is that, once delivered into a cell, the intracellular production of the immunogen favours the induction of MHC class 1-restricted cytotoxic T-cells as compared to other forms of vaccination involving the use of killed whole cell and formulated sub-unit immunogens, which tend to favour the elicitation of MHC class 2-restricted immuno-regulatory responses in the majority of cases studied (ref. 2). In this context, it is, therefore, favourable to use DNA technology to construct naked DNA immunogens for vaccination purposes in order to optimize the induction of cellular effector response against intracellular organisms, such as viruses as well as certain tumours. The other advantages DNA vaccines offer include: (i) the ease to produce them; and (ii) their stability over a wide temperature range.

A common model which has been used recently to predict murine and human CTL antigenic determinants has involved the identification of binding motifs for the respective MHC class 1 molecules from the primary sequences of the native protein molecules (see refs. 3 to 5). Thus, it has been proposed that motifs which are most favoured to bind and lodge into the peptide-binding groove of the H-2D^d gene product is usually 8 to 10 amino acids long. In the majority of cases, these peptides are found to contain anchor residues, such as glycine and proline (GP), at positions 2 and 3 near the amino- (N-) terminus, and either a leucine or phenylalanine at the carboxy- (C-) terminus, which serve to interact with the respective 'pockets' of the peptide-binding groove of a membrane-bound H-2D^d molecule. The motifs restricted to the other class 1 allele, K^d, of the H-2^d haplotype were reported to contain a tyrosine at position 2, and could be an isoleucine, valine or leucine at the C-terminus. Studies of the peptides isolated from the human MHC class 1 molecules, HLA-A0201, had similarly revealed that

the anchor residues were leucine or methionine at position 2 and valine or leucine at the C-terminus in the majority of cases.

The suitability of the HIV-1(BX08) gp140 gene product as a CTL-inducing immunogen was assessed by prediction algorithms to determine the number of both the murine and human MHC class 1-restricted binding motifs it contained. The amino acid sequences of the binding motifs and the designation of the peptides representing them are shown in Table 1 below. Such peptides are novel and are claimed herein. The presence of binding motifs towards the different H-2^d restricted class 1 alleles, i.e. D^d and K^d, allows the immunogenicity of plasmids, pCMV.gp140.BX08, pMP83, pMP84 and pMP88, expressing gp140 of HIV-1 of the primary isolate, BX08, and constructed as described in the Examples below, to be studied in the inbred mouse strain BALB/c of the H-2^d haplotype. The elements and restriction sites of plasmid pCMV.gp140.BX08 are shown in Figure 1. The construction of the plasmids pMP83, pMP84 and pMP88 is described in Example 5 below. The nucleotide sequence (SEQ ID No: 1) and the deduced amino acid sequence (SEQ ID No: 2) of the gp140 open reading frame of the plasmid pCMV.gp140.BX08, pMP83, pMP84 and pMP88, is shown in Figure 2, which appear to be unique sequences and are claimed herein along with their complements.

The location of several binding motifs against the human MHC class 1 allele, HLA-A0201, as seen in Table 1, implied that, under an appropriate immunization regimen, the plasmid has the potential to elicit CTL response directed to these epitopes in the context of this class 1 molecule in human subjects.

The immunogenicity of the plasmid pCMV.gp140.BX08 was studied in BALB/c mice. The results of the study involving three injections of the plasmid at 100.0 μg per dose using the intramuscular route are shown in Figure 3. Upon *in vitro* re-stimulation of the spleenocytes of the plasmid-immunized animals with irradiated autologous LPS blasts pulsed individually with the D^d- and K^d-restricted motif containing peptides, namely, CLP-501 and CLP-504 (SEQ ID Nos.: 3, 5), respectively, it was found that CTLs were generated that killed P815 targets presented with the respective peptides (Figs. 3A and 3B). The amino acid

sequences of the peptides are shown in Table 1. A comparison of the magnitude of the responses at the same effector to target (E:T) ratio revealed that the D^d-restricted response to the CLP-501 peptide is immuno-dominant and that the K^d-restricted response to the CLP-504 peptide is sub-dominant. The *in vitro* re-stimulation leading to the expansion of the effectors was specific because the addition of the same number of irradiated LPS blasts alone (not treated with peptide) did not lead to any generation of effectors in the bulk culture able to kill either of the specific targets tested. The findings that the control group of mice injected with the pCMV vector without the gp140 insert alone failed to generate any of the two sub-populations of CTLs (Fig. 3C) confirmed that the plasmid, pCMV.gp140.BX08, was indeed immunogenic.

The pCMV.gp140.BX08 plasmid, when delivered with the gene gun, was similarly found to be immunogenic. The results shown in Figure 4 show that following two injections at a dose of 0.7 μg of the plasmid, and using the same *in vitro* re-stimulation condition described above that CTLs recognizing the CLP-501 and CLP-504 peptides were detected (Figures 4A and 4B), while no effector response was elicited by the group of animals given the vector, pCMV, alone (Figure 4C).

The immunogenicity of the plasmids pMP83, pMP84 and pMP88 was separately studied, delivered intramuscularly, in BALB/c mice, in comparison to pCMV.gp140.BX08, at three different dosage levels, namely 1.0, 10.0 and 100.0 μg of DNA following the above described procedures. The results obtained are contained in Tables II and III below. CTL activation is achieved at significantly lower doses of the alphavirus vectors than with pCMV.gp140.BX08.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis and treatment of HIV infections. A further non-limiting discussion of such uses is further presented below.

Immunogenic compositions, including vaccines, containing the DNA vector may be prepared as injectables, in physiologically-acceptable liquid solutions or emulsions for polynucleotide administration. The DNA vector may be associated with liposomes, such as lecithin liposomes or other liposomes

known in the art, as a nucleic acid liposome (for example, as described in WO 93/24640) or the DNA vector may be associated with an adjuvant, as described in more detail below.

Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions, such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture up to 100% of the polynucleotide. In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses the degradative enzymes of the lysosomal compartment. Published PCT application WO 94/27435 describes compositions for genetic immunization comprising cationic lipids and polynucleotides.

Agents which assist in the cellular uptake of nucleic acid, such as calcium ions, viral proteins and other transfection facilitating agents, may advantageously be used with the vector.

Polynucleotide immunogenic preparations may also be formulated as microcapsules, including biodegradable time-release particles. Thus, U.S. Patent 5,151,264 describes a particular carrier of a phospholipid/glycolipid/ polysaccharide nature that has been termed Bio Vecteurs Supra Moléculaires (BVSM). The particulate carriers are intended to transport a variety of molecules having biological activity in one of the layers thereof.

U.S. Patent 5,075,109 describes encapsulation of the antigens trinitrophenylated keyhole limpet hemocyanin and staphylococcal enterotoxin B in 50:50 poly (DL-lactideco-glycolide). Other polymers for encapsulation are suggested, such as poly(glycolide), poly(DL-lactide-co-glycolide), copolyoxalates, polycaprolactone, poly(lactide-co-caprolactone), poly(esteramides), polyorthoesters and poly(8-hydroxybutyric acid), and polyanhydrides.

Published PCT application WO 91/06282 describes a delivery vehicle comprising a plurality of bioadhesive microspheres and antigens. The microspheres being of starch, gelatin, dextran, collagen or albumin. This delivery vehicle is particularly intended for the uptake of vaccine across the nasal mucosa. The delivery vehicle may additionally contain and absorption enhancer.

In particular embodiments of the present invention, the vector may be delivered in conjunction with a targeting molecule to target the vector to selected cells including cells of the immune system.

The vectors may be delivered to the host by a variety of procedures, for example, Tang et al (ref. 6) discloses that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice, while Furth et al. (ref. 7) showed that a jet injector could be used to transfect skin, muscle, fat and mammary tissues of living animals.

Biological Deposits

Certain vectors that contain nucleic acid coding for an extracellular fragment of gp140 of a primary isolate that are described and referred to herein as well as precursor alphavirus vectors have been deposited with the America Type Culture Collection (ATCC) located at 10801 University Boulevard, Manassas, Virginia 20110-2209, USA, pursuant the Budapest Treaty and prior to the filing of this application. Samples of the deposited vectors will become available to the public and all restrictions imposed or access to the deposits will be received upon grant of a patent based on this United States patent application. In addition, the deposit will be replaced if viable samples cannot be dispensed by the Depository. The invention described and claimed herein is not limited in scope by the biological materials deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar vectors that contain nucleic acid which encodes equivalent or similar antigens as described in this application are within the scope of the invention.

Deposit Summary

Plasmid	ATCC	Deposited Date
pCMV.gp140.BX08	203839	March 9, 1999
pMP42	203461	November 12, 1998
pMP76	203462	November 12, 1998

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific

Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

Methods of plasmid construction, peptide synthesis, cell culture, CTL assay and other testing procedures that are not explicitly described in this disclosure are amply reported in the scientific literature and are well within the scope of those skilled in the art.

Example 1

This Example illustrates the construction of the plasmid, pCMV.gp140.BX08.

The plasmid, pCMV.gp140.BX08, contains sequence segments from various sources, and the elements of construction are depicted in Figure 1. The nucleotide (SEQ ID No: 1) and derived amino acid sequences (SEQ ID No: 2) of the gp140 open reading frame of the plasmid are shown in Figure 2.

The prokaryotic vector pBluescript SK (Stratagene) is the backbone of the plasmid pCMV.gp140.BX08 and was modified by the replacement of the Amp^R with Kan^R gene and the deletion of the f1 and the LacZ regions. To achieve the desired modifications, the sequence between AhdI (nucleotide 2,041) and SacI (nucleotide 759) of pBluescript SK, which contains the Amp^R, f1 origin and the LacZ, was deleted. A 1.2 kb PstI fragment from the plasmid pUC-4K (Pharmacia) containing the Kan^R gene, was blunt end ligated to the AhdI site of pBluescript SK in a counter-clockwise orientation relative to it's transcription. A 1.6 kb SspI/PstI DNA fragment containing the human cytomegalovirus immediate-early gene promotor, enhancer and intron A sequences (CMV) was ligated to the other end of the Kan^R gene so that the transcription from the CMV promotor proceeds in the clockwise orientation. A synthetic oligonucleotide segment containing translation initiation sequence and sequences encoding the human tissue plasminogen activator signal peptide (TPA) was used to link the CMV promotor and the sequences encoding the gp140 of the primary isolate HIV-1_{BX08}. The gp140 sequence encodes a portion of the envelope protein between amino acid 33 and

666 which ends before the transmembrane domain of gp41 (see Figure 2). A translation termination codon was placed at the end of the gp140 coding sequence. Next to the gp140 coding region is a 0.2 kb fragment containing the bovine growth hormone (BGH) polyadenylation signal sequence that is PCR amplified from pRC/CMV (Invitrogen). A remnant 80 bp DNA segment from the SV40 polyadenylation signal remained between the BGH poly A sequence and the SacI site of pBluescript SK due to DNA manipulation and it serves no purpose in this plasmid.

The pCMV.gp140.BX08 construct was introduced into HB101 competent cells according to manufacturer's recommendations (GibcoBRL). Correct molecular clones were identified by restriction and sequencing analysis and their expression of gp140 was examined in transient transfections followed by Western blot analysis.

All DNAs used for immunizations were prepared using EndoFree Plasmid Kit (Qiagen). For intramuscular immunizations in mice, 100 μg of pCMV.gp140.BX08, in 100 μl PBS was injected into the tibialis anterior muscles at 4 weeks intervals. Gene gun immunizations were accomplished with the Helios Gene Gun System (Biorad). Cartridges were prepared according to manufacturer's recommendations. Specifically, each cartridge was made to contain 0.7 μg of the DNA and 0.5 mg gold. Immunizations were carried out by applying two cartridges to each animal onto the shaved abdominal area at 4 week intervals.

Example 2

This Example illustrates the synthesis of peptides.

Solid phase peptide synthesis of peptide CLP-501 and CLP-504 were conducted on an ABI 430A automated peptide synthesizer according to the manufacturer's standard protocols. The peptides were cleaved from solid support by treatment with liquid hydrogen fluoride in the presence of thiocresole, anisole, and methyl sulfide. The crude products were extracted with trifluoroacetic acid (TFA) and precipitated with diethyl ether.

The amino acid sequences of these peptides are shown in Table 1.

Example 3

This Example illustrates *in vitro* cell culture protocols to re-stimulate and expand CTLs and assay for their effector functions.

Spleens of BALB/c mice injected with the plasmid, pCMV.gp140.BX08, prepared and formulated as described in Example 1, using the intramuscular route or gene gun delivery method, were removed 10 to 11 days post final booster injection. Spleenocytes at 3.0 x 10^7 were co-cultured with 1.3 x 10^7 autogolous LPS blasts which had been pulsed with the test peptide for 5hr at 37°C and irradiated at 3000 rads in 10.0 ml of complete medium (RPMI 1640 supplemented with 10.0% 56°C heat-inactivated bovine serum, 120.0 units per ml of penicillin G sodium, 120.0 µg per ml of streptomycin sulphate and 0.35 mg per ml of L-glutamine) in a 25 cm^2 tissue culture flask. The cultures were kept at 37°C in a humidified CO_2 incubator for days, and the responders were then tested against peptide-pulsed P815 target cells in a standard *in vitro* 4 hr CTL assay as follows:

The responders were harvested from the 7-day cultures and washed once with RPMI 1640 medium without added bovine serum. The positive target was created by incubating 3 to 5 x 10^6 P815 cells with 100.0 µg of the specified peptide overnight in a 26°C water bath. The target cells were then labeled with ^{51}Cr at 250.0 uCi per 1 x 10^6 cells in the presence of 25.0 µg of the same test peptides for 60 to 75 minutes at 26°C. After washing twice with complete medium to remove excess ^{51}Cr, the targets were incubated at 2.5 x 10^3 with different numbers of the responders per well in a V-bottomed 96 will tissue culture plates for 4 hr in a 37°C $C0_2$ incubator. Half amount of the supernatant from each micro-assay culture was then removed and counted for radioactivity. Results were expressed as % which was calculated using the equation:

% lysis = (spontaneous lysis in cpm of experimental sample – spontaneous lysis in cpm of labeled target cells alone) divided by (total lysis in cpm of target cells alone – spontaneous lysis in cpm of target cells alone) x 100.

The results obtained employing intramuscular injection are shown in Figures 3A, 3B and 3C while those obtained employing the gene gun delivery are shown in Figures 4A, 4B and 4C.

Example 4

This Example illustrates the construction of alphavirus expression vectors pMP83, pMP84 and pMP88.

DNA vector pCMV.gp140.BX08, prepared as described in Example 1, was digested with restriction endonucleases NbeI and BamHI to release the HIV-1 gp140 sequence. The NheI/BamHI fragment was gel purified and ligated to a synthetic oligonucleotide linker made from the following annealed oligonucleotides: Oligo 1 - TPA-1

5'- TCC GGA TCC ACC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG G -3' (SEQ ID No: 16)

Oligo 2 - TPA-2

5'- CTA GTC GAA ACG AAG ACT GCT CCA CAC AGC AGC AGC ACA CAG CAG AGC CCT CTC TTC ATT GCA TCC ATG GTG GAT CCG GA -3' (SEQ ID No: 17). This ligation restrored the TPA signal sequence. The resulting fragment was restricted with BamHI and the resulting BamHI fragment was gel purified. The nucleotide sequence of the BamHI fragment is shown in Figure 5 (SEQ ID No: 15).

After the DNA sequence verification, the BamHI fragment was cut out of the pUC19 vector and ligated into BamHi restricted plasmids pMP42, pMP74 and pMP76 to make plasmids pMP88, pMP84 and pMP83 respectively.

Plasmid pMP42, containing the SFV replicon, is described in WO 99/25858 (1038-864), assigned to the applicants and the disclosure of which is incorporated herein by reference and has been deposited with ATCC (203461). The construction of pMP42 is shown in Figures 2A and 2B of WO 99/25858.

Plasmid pMP76, containing the SFV replicon, is described in WO 99/25859 (1038-862), assigned to the applicants and the disclosure of which is incorporated herein by reference and has been deposited with ATCC (203462). The construction of pMP76 is shown in Figures 8A to 8D of WO 99/25859.

Plasmid pMP74, containing the SFV replicon, is identical to pMP76 except that it lacks the rabbit β-globin intron II insertion into the SFV replicon. This plasmid may be constructed by suitable modification to the scheme shown in Figures 8A to 8D of WO 99/25859.

Example 5

This Example shows the results of immunizations using the alphavirus vectors.

The recombinant alphavirus vectors pMP88, pMP84 and pMP83, prepared as described in Example 4, were employed in comparative immunogenicity studies with plasmid pCMV.gp140.BX08, prepared as described in Example 1, in BALB/c mice following the procedure outlines in Example 1 for intramuscular immunization in mice using pCMV.gp140.BX08 and the CTL assay of Example 3, with unmodified pMP76 and pCMV being employed as negative controls. The results obtained are shown in Table II below.

Comparative analysis of the alphavirus constructs and the DNA construct of Example 1 showed similar results in the CTL assay. As expected, the negative control vectors that did not contain the gp140 sequences from HIV-1 BX08 showed no specific lysis in the CTL assay. All three alphavirus replicons, pMP83, pMP84 and pMP88, showed specific lysis as did the vector pCMV.gp140.BX08. The difference between the two types of vector was the amount of immunizing nucleic acid needed to elicit the same response. At 1 μg dose, the alphavirus vectors pMP83 and pMP88 showed comparable responses to pCMV.gp140.BX08 at a much higher dose of 100 μg.

These results were confirmed by an vector from a interferon-gamma (IFN-γ) assay, the results of which are shown in Table III. The assay is well known, as the measure of IFN-γ secreted from the spleenocytes indicated activation of the CTLs. Again, the alphavirus vectors showed comparable activation at an approximately 100 fold lower dose than the pCMV.gp140.BX08 vector. Overall these results indicate that immunization with nucleic acid vector expressing the gp140 sequence from the primary isolate BX08 generated MHC Class I restricted cytotoxic T-cells and that the alphavirus expression system used was approximately 100-fold more effective at the lower dose.

SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention provides a novel plasmid expressing, *in vitro* and *in vivo*, the gp140 protein of the primary HIV-1

isolate BX08 and the generation of MHC class 1-restricted cytotoxic T-cells in animals. Modifications are possible within the scope of this invention.

Table 1

MHC class 1-restricted motifs of the extracellular envelope fragment, gp140, of HIV-1(BX08)

H-2^d-restricted *				HLA-A0201-restricted **
Peptide ***	D^d-	Peptide ***	K^d-	
CLP-501	IGPGRAFYTT (274-283) (SEQ ID No:3)	CLP-503	AYDTEVHNV (29-37) (SEQ ID No:4)	KLTPLCVTL (91-98) (SEQ ID No:10)
		CLP-504	FYSLKIVPI (141-149) (SEQ ID No:5)	TLFRVAIKL (305-313) (SEQ ID No:11)
		CLP-505	LYKYKVVKI (443-451) (SEQ ID No:6)	TLTVQARQL (403-411) (SEQ ID No:12)
		CLP-506	KYKVVKIEPL (445-454) (SEQ ID No:7)	TLTVQARAL (496-504) (SEQ ID No:13)
		CLP-507	RYLQDQRFL (545-553) (SEQ ID No:8)	QLQARVLAL (535-543) (SEQ ID No:14)
		CLP-508	NYTEIIYSL (597-605) (SEQ ID No:9)	

* or ** Anchors residues were typed in bolded letters.

*** Peptides chosen for the study reported herein are bolded.

Table II

A comparative immunogenicity study of recombinant alpha viruses and pCMV.gp140.BX08 expressing gp140 of HIV-1_{BX08} in BALB/c mice

Immunization (3 injections, im)	Dose (μg)	% CLP-501 specific lysis at E:T ratio of 75:1
pMP76	100.0 10.0 1.0	0.6 0.02 0
pMP83	100.0 10.0 1.0	23.4 38.5 42.7
pMP84	100.0 10.0 1.0	37.2 18.6 12.4
pMP88	100.0 (not done, mice died) 10.0 1.0	 39.6 49.6
pCMV3	100.0 10.0 1.0	0 0.06 0
PCMV.gp140.BX08	100.0 10.0 1.0	59.7 29.3 0

Table III

Frequencies of spleenic CLP-501-specific effectors induced by recombinant vectors expressing HIV-1_{BX08} gp140 determined by IFN-gamma ELISPOT

Immunization (3 injections, im)	Dose (μg)	Number of spots scored per $5X10^5$ responders In duplicate cultures
pMP76	100.0 10.0 1.0	0, 0 0, 0 0, 0
pMP83	100.0 10.0 1.0	10, 14 14, 19 18, 13
pMP84	100.0 10.0 1.0	7, 12 4, 7 2, 6
pMP88	100.0 (not done, mice died) 10.0 1.0	 20, 13 24, 17
pCMV3	100.0 10.0 1.0	0 0 0
PCMV.gp140.BX08	100.0 10.0 1.0	17, 13 6, 9 0

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CLAIMS

What we claim is:

1. A vector, comprising a gene encoding the extracellular fragment gp140 of the primary HIV-1 isolate BX08 under the control of a promotor for expression of the gene product in a host organism.

2. The vector of claim 1, wherein the promoter is the cytomegalovirus promotor.

3. The vector of claim 1, which has the identifying characteristics of pCMV.gp140.BX08 (ATCC No. 203839), as shown in Figure 1.

4. The vector of claim 1, which has the identifying characteristic of pMP83, pMP84 or pMP88

5. An immunogenic composition comprising a vector comprising a gene encoding the extracellular fragment gp140 of the primary HIV-1 isolate BX08 under the control of a promotor for expression of the gene product in a host organism.

6. The immunogenic compositions of claim 5, wherein the promoter is the cytomegalovirus promotor.

7. The immunogenic composition of claim 5, wherein the vector is pCMV.gp140.BX08.

8. The immunogenic composition of claim 5 wherein the vector is pMP83, pMP84 or pMP88.

9. The immunogenic composition of claim 5 formulated for intramuscular immunization with a pharmaceutically-acceptable liquid carrier.

10. The immunogenic composition of claim 6 formulated for gene gun delivery with gold particles.

11. A method of generating a cytotoxic T-cell response to HIV-1 in a host, which comprises administering to the host the immunogenic composition of claim 5

12. The vector according to claim 1 when used as an immunogen for generating a cytotoxic T-cell response to HIV-1 in a host.

13. The use of a vector according to claim 1 in the manufacture of an immunogen for generating a cytotoxic T-cell response to HIV1 in a host.

14. A peptide consisting of an amino acid sequence selected from the group consisting of SEQ ID Nos.: 4 to 14, as shown in Table 1.

15. The peptide of claim 14, consisting of SEQ ID No.:5.

16. A nucleic acid molecule consisting of a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence consisting of SEQ ID No: 1 or the complementary sequence thereto;

(b) a nucleotide sequence encoding a gp140 protein consisting of SEQ ID No: 2 or the complementary sequence thereto; and

(c) a nucleotide sequence consisting of SEQ ID No: 15 or the complementary sequence thereto.

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(71) Applicant *(for all designated States except US)*: CONNAUGHT LABORATORIES LIMITED [CA/CA]; 1755 Steeles Avenue West, Toronto, Ontario M2R 3T4 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: SIA, Charles, D., Y. [CA/CA]; 189 Mabley Crescent, Thornhill, Ontario L4J 2Z7 (CA). CAO, Shi-Xian [CA/CA]; Apt. 408, 716 The West Mall, Etobicoke, Ontario M9C 4X6 (CA). PERSSON, Roy [CA/CA]; Unit 604, 7 Bishop Avenue, North York, Ontario M2M 4J4 (CA). ROVINSKI, Benjamin [CA/CA]; 70 Winding Lane, Thornhill, Ontario L4J 5H6 (CA). PARRINGTON, Mark [CA/CA]; 45 Martin Street, Bradford, Ontario L3Z 1Z4 (CA).

(74) Agent: STEWART, Michael, L.; Sim & Mcburney, 6th Floor, 330 University Avenue, Toronto, Ontario M5G 1R7 (CA).

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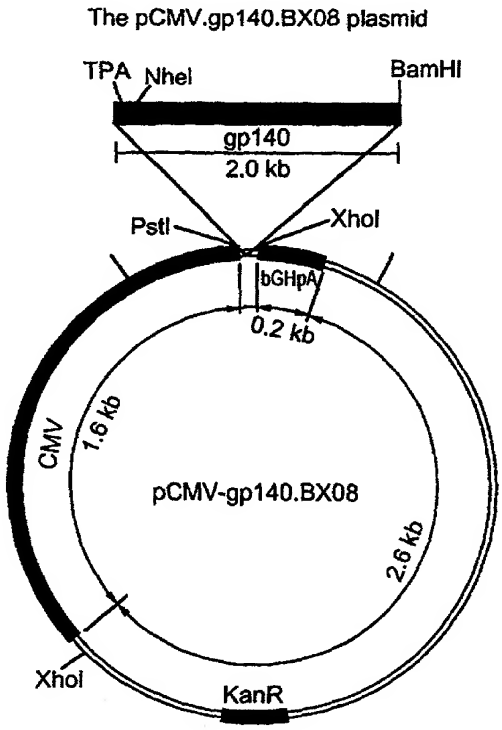
(54) Title: EXPRESSING GP140 FRAGMENT OF PRIMARY HIV-1 ISOLATE

(57) Abstract

A vector for eliciting an immune response to a host comprising a gene encoding the gp140 protein of the primary isolate of HIV-1, BX08, under the control of a promotor for expression of the protein in the host, specifically plasmids pCMV.gp140.BX08, pMP83, pMP84 and pMP88. Murine and human MHC class 1-restricted binding motifs contained in BX08 are identified.



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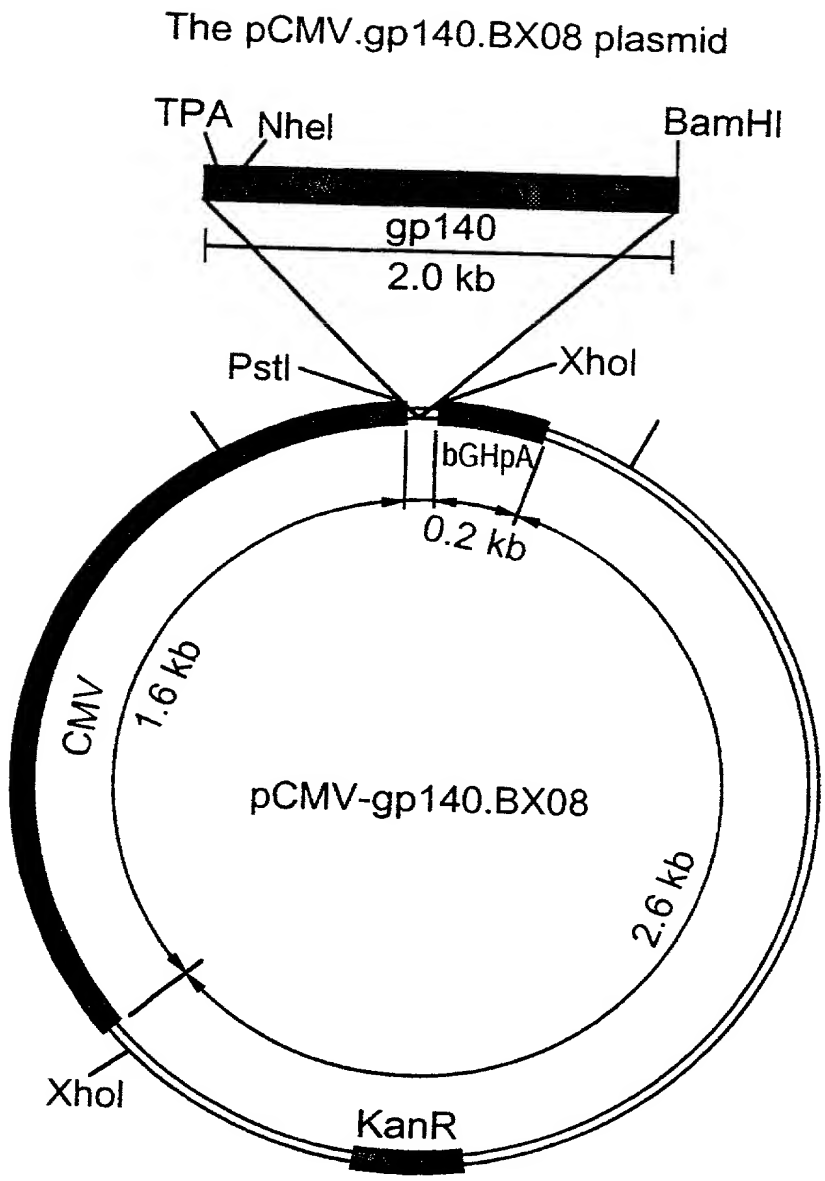
The pCMV.gp140.BX08 plasmid
TPA
NheI
BamHI
gp140
2.0 kb
PstI
XhoI
bGHpA
0.2 kb
CMV
1.6 kb
pCMV-gp140.BX08
2.6 kb
XhoI
KanR

FIG.1

FIG.2A

```
+1  M  D  A  M  K  R  G  L  C  C  V  L  L  L  C  G
1   ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG CTG TGT GGA
    TAC CTA CGT TAC TTC TCT CCC GAG ACG ACA CAC GAC GAC GAC ACA CCT

+1  A  V  F  V  S  A  S  L  W  V  T  V  Y  Y  Y  G  V
49  GCA GTC TTC GTT TCG GCT AGC TTG TGG GTC ACA GTC TAT TAT GGG GTA
    CGT CAG AAG CAA AGC CGA TCG AAC ACC CAG TGT CAG ATA ATA CCC CAT

+1  P  V  W  K  E  A  T  T  T  L  F  C  A  S  D  A
97  CCT GTG TGG AAA GAA GCA ACC ACC ACT CTA TTT TGT GCA TCA GAT GCT
    GGA CAC ACC TTT CTT CGT TGG TGG TGA GAT AAA ACA CGT AGT CTA CGA

+1  K  A  Y  D  T  E  V  H  N  V  W  A  T  H  A  C
145 AAA GCA TAT GAT ACA GAA GTA CAT AAT GTT TGG GCC ACA CAT GCC TGT
    TTT CGT ATA CTA TGT CTT CAT GTA TTA CAA ACC CGG TGT GTA CGG ACA

+1  V  P  T  D  P  N  P  Q  E  V  V  L  G  N  V  T
193 GTA CCC ACA GAC CCC AAC CCA CAA GAA GTA GTA TTG GGA AAT GTG ACA
    CAT GGG TGT CTG GGG TTG GGT GTT CTT CAT CAT AAC CCT TTA CAC TGT

+1  E  N  F  N  M  G  K  N  N  M  V  E  Q  M  H  E
241 GAA AAT TTT AAC ATG GGG AAA AAT AAC ATG GTA GAA CAG ATG CAT GAA
    CTT TTA AAA TTG TAC CCC TTT TTA TTG TAC CAT CTT GTC TAC GTA CTT

+1  D  I  I  S  L  W  D  Q  S  L  K  P  C  V  K  L
289 GAT ATA ATT AGT TTA TGG GAT CAA AGC CTA AAG CCA TGT GTA AAA TTA
    CTA TAT TAA TCA AAT ACC CTA GTT TCG GAT TTC GGT ACA CAT TTT AAT
```

FIG.2B

```
      +1  T  P  L  C  V  T  L  N  C  T  K  L  K  N  S  T
     337  ACC CCA CTC TGT GTT ACT TTA AAT TGC ACT AAG TTG AAG AAT AGT ACT
          TGG GGT GAG ACA CAA TGA AAT TTA ACG TGA TTC AAC TTC TTA TCA TGA

      +1  D  T  N  N  T  R  W  G  T  Q  E  M  K  N  C  S
     385  GAT ACC AAT AAT ACT AGA TGG GGA ACA CAA GAA ATG AAA AAC TGC TCT
          CTA TGG TTA TTA TGA TCT ACC CCT TGT GTT CTT TAC TTT TTG ACG AGA

      +1  F  N  I  S  T  S  V  R  N  K  M  K  R  E  Y  A
     433  TTC AAC ATC AGC ACA AGT GTA AGA AAT AAG ATG AAG AGA GAA TAT GCA
          AAG TTG TAG TCG TGT TCA CAT TCT TTA TTC TAC TTC TCT CTT ATA CGT

      +1  L  F  Y  S  L  D  I  V  P  I  D  N  D  N  T  S
     481  CTT TTT TAT AGT CTT GAT ATA GTA CCA ATA GAT AAT GAT AAT ACT AGC
          GAA AAA ATA TCA GAA CTA TAT CAT GGT TAT CTA TTA CTA TTA TGA TCG

      +1  Y  R  L  R  S  C  N  T  S  I  I  T  Q  A  C  P
     529  TAT AGG TTA AGA AGT TGT AAT ACC TCA ATC ATT ACA CAG GCC TGT CCA
          ATA TCC AAT TCT TCA ACA TTA TGG AGT TAG TAA TGT GTC CGG ACA GGT

      +1  K  V  S  F  E  P  I  P  I  H  F  C  A  P  A  G
     577  AAG GTA TCC TTT GAG CCA ATT CCC ATA CAT TTT TGT GCC CCG GCT GGT
          TTC CAT AGG AAA CTC GGT TAA GGG TAT GTA AAA ACA CGG GGC CGA CCA

      +1  F  A  I  L  K  C  N  N  K  T  F  N  G  T  G  P
     625  TTT GCG ATT CTA AAG TGT AAC AAT AAA ACG TTC AAT GGA ACA GGA CCA
          AAA CGC TAA GAT TTC ACA TTG TTA TTT TGC AAG TTA CCT TGT CCT GGT
```

FIG.2C

```
    +1 C   T   N   V   S   T   V   Q   C   T   H   G   I   R   P   V
673    TGT ACA AAT GTC AGC ACA GTA CAA TGT ACA CAT GGA ATT AGG CCA GTA
       ACA TGT TTA CAG TCG TGT CAT GTT ACA TGT GTA CCT TAA TCC GGT CAT

    +1 V   S   T   Q   L   L   L   N   G   S   L   A   E   E   E   V
721    GTA TCA ACT CAA CTG CTG TTA AAT GGC AGC CTA GCA GAA GAA GAG GTA
       CAT AGT TGA GTT GAC GAC AAT TTA CCG TCG GAT CGT CTT CTT CTC CAT

    +1 V   I   R   S   E   N   F   T   N   N   A   K   T   I   I   V
769    GTA ATT AGA TCT GAA AAT TTC ACA AAC AAT GCT AAA ACC ATA ATA GTA
       CAT TAA TCT AGA CTT TTA AAG TGT TTG TTA CGA TTT TGG TAT TAT CAT

    +1 Q   L   N   E   S   V   E   I   N   C   T   R   P   N   N   N
817    CAG CTA AAT GAA TCT GTA GAA ATT AAT TGT ACA AGA CCC AAC AAC AAT
       GTC GAT TTA CTT AGA CAT CTT TAA TTA ACA TGT TCT GGG TTG TTG TTA

    +1 T   R   K   S   I   H   I   G   P   G   R   A   F   Y   T   T
865    ACA AGA AAA AGT ATA CAT ATA GGA CCA GGG AGA GCA TTT TAT ACA ACA
       TGT TCT TTT TCA TAT GTA TAT CCT GGT CCC TCT CGT AAA ATA TGT TGT

    +1 G   D   I   I   G   D   I   R   Q   A   H   C   N   I   S   R
913    GGA GAT ATA ATA GGA GAT ATA AGA CAA GCA CAT TGT AAC ATT AGT AGA
       CCT CTA TAT TAT CCT CTA TAT TCT GTT CGT GTA ACA TTG TAA TCA TCT
    +1 T   N   W   T   N   T   L   K   R   V   A   E   K   L   R   E
961    ACA AAC TGG ACT AAC ACT TTA AAA AGG GTA GCT GAA AAA TTA AGA GAA
       TGT TTG ACC TGA TTG TGA AAT TTT TCC CAT CGA CTT TTT AAT TCT CTT
```

FIG.2D

```
+1  K  F  N  N  T  T  I  V  F  N  Q  S  S  G  G  D
1009 AAA TTT AAT AAT ACA ACA ATA GTC TTT AAT CAA TCC TCA GGA GGG GAC
     TTT AAA TTA TTA TGT TGT TAT CAG AAA TTA GTT AGG AGT CCT CCC CTG

+1  P  E  I  V  M  H  S  F  N  C  G  G  E  F  F  Y
1057 CCA GAA ATT GTA ATG CAC AGT TTT AAT TGT GGA GGG GAA TTT TTC TAC
     GGT CTT TAA CAT TAC GTG TCA AAA TTA ACA CCT CCC CTT AAA AAG ATG

+1  C  N  T  T  Q  L  F  N  S  T  W  N  E  T  N  S
1105 TGT AAT ACA ACA CAA CTG TTT AAT AGT ACT TGG AAT GAA ACT AAC AGT
     ACA TTA TGT TGT GTT GAC AAA TTA TCA TGA ACC TTA CTT TGA TTG TCA

+1  E  G  N  I  T  S  G  T  I  T  L  P  C  R  I  K
1153 GAA GGA AAT ATC ACC AGT GGA ACT ATA ACA CTC CCA TGC AGA ATA AAA
     CTT CCT TTA TAG TGG TCA CCT TGA TAT TGT GAG GGT ACG TCT TAT TTT

+1  Q  I  I  N  M  W  Q  E  V  G  K  A  M  Y  A  P
1201 CAA ATT ATA AAC ATG TGG CAG GAA GTA GGA AAA GCA ATG TAT GCC CCT
     GTT TAA TAT TTG TAC ACC GTC CTT CAT CCT TTT CGT TAC ATA CGG GGA

+1  P  I  G  G  Q  I  K  C  L  S  N  I  T  G  L  L
1249 CCC ATC GGA GGA CAA ATT AAA TGT TTG TCA AAC ATC ACA GGG CTG TTA
     GGG TAG CCT CCT GTT TAA TTT ACA AAC AGT TTG TAG TGT CCC GAC AAT

+1  L  T  R  D  G  G  S  D  N  S  S  S  G  K  E  I
1297 TTA ACA AGA GAT GGT GGT AGT GAT AAC AGT AGT AGT AGT GGG AAA GAG ATC
     AAT TGT TCT CTA CCA CCA TCA CTA TTG TCA TCA TCA CCC TTT CTC TAG
```

FIG.2E

```
+1  F  R  P  G  G  G  D  M  R  D  N  W  R  S  E  L
1345 TTC AGA CCT GGA GGG GGA GAT ATG AGG GAC AAT TGG AGA AGT GAA TTA
     AAG TCT GGA CCT CCC CCT CTA TAC TCC CTG TTA ACC TCT TCA CTT AAT

+1  Y  K  Y  K  V  V  K  I  E  P  L  G  I  A  P  T
1393 TAT AAA TAT AAG GTA GTA AAA ATT GAA CCA TTA GGA ATA GCA CCC ACC
     ATA TTT ATA TTC CAT CAT TTT TAA CTT GGT AAT CCT TAT CGT GGG TGG

+1  K  A  K  R  R  V  V  Q  R  E  K  R  A  V  G  I
1441 AAG GCA AAG AGA AGA GTG GTG CAG AGA GAA AAA AGA GCA GTG GGA ATA
     TTC CGT TTC TCT TCT CAC CAC GTC TCT CTT TTT TCT CGT CAC CCT TAT

+1  G  A  M  F  L  G  F  L  G  A  A  G  S  T  M  G
1489 GGA GCC ATG TTC CTT GGG TTC TTG GGA GCA GCA GGA AGC ACT ATG GGC
     CCT CGG TAC AAG GAA CCC AAG AAC CCT CGT CGT CCT TCG TGA TAC CCG

+1  A  A  S  L  T  L  T  V  Q  A  R  Q  L  L  S  G
1537 GCA GCG TCA CTA ACG CTG ACG GTA CAG GCC AGA CAA TTA TTG TCT GGT
     CGT CGC AGT GAT TGC GAC TGC CAT GTC CGG TCT GTT AAT AAC AGA CCA

+1  I  V  Q  Q  Q  N  N  L  L  R  A  I  E  A  Q  Q
1585 ATA GTG CAG CAG CAA AAC AAT TTG CTG AGG GCT ATT GAG GCG CAA CAG
     TAT CAC GTC GTC GTT TTG TTA AAC GAC TCC CGA TAA CTC CGC GTT GTC

+1  H  L  L  Q  L  T  V  W  G  I  K  Q  L  Q  A  R
1633 CAC CTG TTG CAA CTC ACA GTC TGG GGC ATC AAG CAG CTC CAG GCA AGA
     GTG GAC AAC GTT GAG TGT CAG ACC CCG TAG TTC GTC GAG GTC CGT TCT
```

FIG.2F

```
   +1  V   L   A   L   E   R   Y   L   Q   D   Q   R   F   L   G   M
1681  GTC CTG GCT CTG GAA AGA TAC CTA CAG GAT CAA CGG TTC CTA GGG ATG
      CAG GAC CGA GAC CTT TCT ATG GAT GTC CTA GTT GCC AAG GAT CCC TAC

   +1  W   G   C   S   G   K   L   I   C   T   T   A   V   P   W   N
1729  TGG GGT TGC TCT GGA AAA CTC ATC TGC ACC ACT GCT GTG CCT TGG AAT
      ACC CCA ACG AGA CCT TTT GAG TAG ACG TGG TGA CGA CAC GGA ACC TTA

   +1  A   S   W   S   N   K   N   L   S   Q   I   W   D   N   M   T
1777  GCT AGT TGG AGT AAT AAA AAT CTA AGT CAG ATT TGG GAT AAC ATG ACC
      CGA TCA ACC TCA TTA TTT TTA GAT TCA GTC TAA ACC CTA TTG TAC TGG

   +1  W   M   E   W   E   R   E   I   S   N   Y   T   E   I   I   Y
1825  TGG ATG GAG TGG GAG AGA GAA ATA AGC AAT TAC ACA GAG ATA ATA TAT
      ACC TAC CTC ACC CTC TCT CTT TAT TCG TTA ATG TGT CTC TAT TAT ATA

   +1  S   L   I   E   E   S   Q   N   Q   Q   E   K   N   E   L   D
1873  AGC TTA ATT GAA GAA TCG CAG AAC CAA CAA GAA AAG AAT GAA CTA GAC
      TCG AAT TAA CTT CTT AGC GTC TTG GTT GTT CTT TTC TTA CTT GAT CTG

   +1  L   L   Q   L   D   K   W   A   S   L   W   N   W   F   D   I
1921  TTA TTA CAA TTG GAT AAG TGG GCA AGT TTG TGG AAT TGG TTT GAC ATA
      AAT AAT GTT AAC CTA TTC ACC CGT TCA AAC ACC TTA ACC AAA CTG TAT

   +1  T
1969  ACA
      TGT
```

Note: shown is the translation of the sense strain with it's anti-sense strain below it that was cloned into the pCMV3 vector described in the text.

Effector responses elicited by intramuscular injection of the plasmid, pCMV.gp140.BX08, into BALB/c mice

Immunization:	100.0 ug pCMV.gp.BX08 + 100.0 ug pCMV	100.0 ug pCMV.gp140.BX08 100.0 ug pCMV	100.0 ug pCMV
In vitro re-stimulation:	with autologous CLP-501 pulsed LPS blasts (■)	with autologous CLP-504 pulsed LPS blasts (●)	with autologous CLP-501 pulsed LPS blasts (▲)
	with autologous LPS Blasts (▲)		with autologous CLP-504 pulsed LPS blasts (●)
Target:	CLP-501-pulsed P815	CLP-504-pulsed P815	CLP-501-pulsed P815 for CLP-501-pulsed blast re-stimulation
			CLP-504-pulsed P815 for CLP-504-pulsed blast re-stimulation

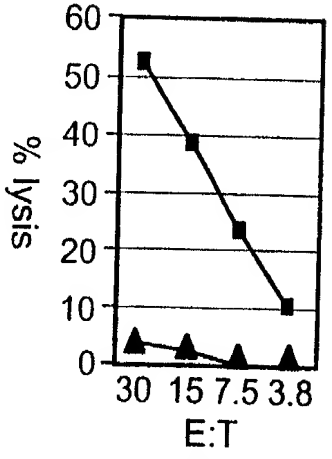

FIG.3A

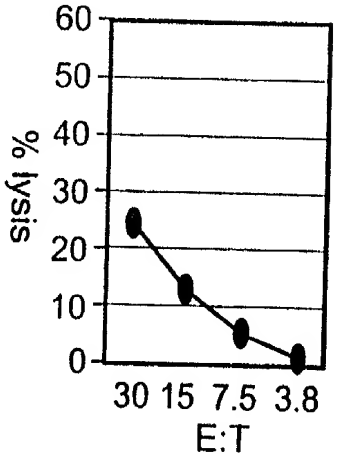

FIG.3B

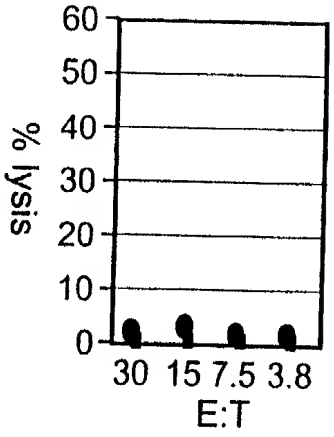

FIG.3C

Effector responses elicited by gene gun delivery of the plasmid, pCMV.gp140.BX08, into BALB/c mice

Immunization:	0.7 ug pCMV.gp140.BX08 + 0.7 ug pCMV	0.7 ug pCMV.gp140.BX08 + 0.7 ug pCMV	1.4 ug pCMV
In vitro re-stimulation:	with autologous CLP-501 pulsed LPS blasts (■)	with autologous CLP-504 pulsed LPS blasts (●)	with autologous CLP-501 pulsed LPS blasts (■)
	with autologous LPS Blasts (▲)		with autologous CLP-504 pulsed LPS blasts (●)
Target:	CLP-501-pulsed P815	CLP-504-pulsed P815	CLP-501-pulsed P815 for CLP-501-pulsed blast re-stimulation
			CLP-504-pulsed P815 for CLP-504-pulsed blast re-stimulation

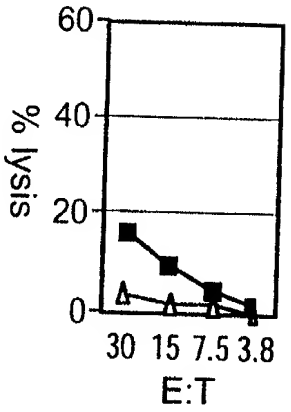

FIG.4A

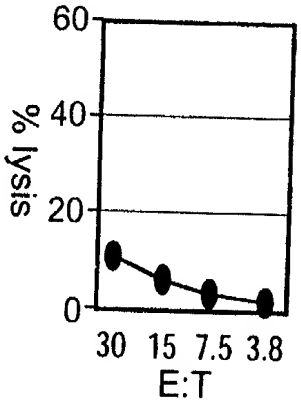

FIG.4B

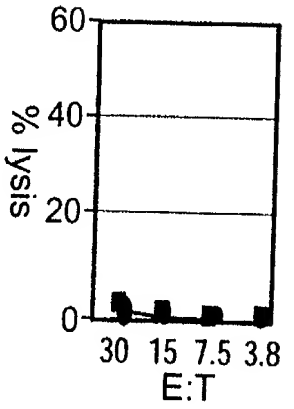

FIG.4C

Sequence of Bam HI fragment containing HIV-1 gp 140 sequences for alphavirus expression vectors.

gatccaccatggatgcaatgaagagagggctctgctgtgtgctgctgttcgttcggctagcttgtgg
gtcacagtctattatggggtacctgtgtggaaagaagcaaccacctctatttgtgcatcagatgctaaagcatatgatac
agaagtacataatgtttgggccacacatgcctgtgtacccacagaccccaacccacaagaagtagtattgggaaatgtga
cagaaaatttaacatggggaaaataacatggtagaacagatgcatgaagatataattagttatgggatcaaagccta
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tggggaacacaagaaatgaaaaactgctctttcaacatcagcacaagtgtaagaaaataagatgaagagagaatatgca
ctttttatagtcttgatatagtaccaatagataatgatacatagctatagattaagaagttgtaatacctcaatcattacaca
ggcctgtccaaaggtatcctttgagccaattcccatacatttttgtgccccggctggtttgcgattctaaagtgtaacaataaa
acgttcaatggaacaggaccatgtacaaaatgtcagcacagtacaatgtacacatggaattaggccagtagtatcaactca
actgctgttaaatggcagcctagcagaagaagaggtagtaattagatctgaaaatttcacaaacaatgctaaaaccataa
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agggagagcatttatacaacaggagatataaatagagacaagcacattgtaacattagtagaacaaaactgg
actaacacttaaaaaggtagctgaaaaattaagagaaaaatttaataaatacaacaatagtcittaatcaatcctcagga
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gtggcaggaagtaggaaaagcaatgtatgcccctcccatcggaggacaaattaaatgtttgtcaaacatcacagggctgt
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ggaatgctagttggagtaataaaatctaagtcagatttgggataacatgacctggatgggagagaaataag
caattacacagagataatatatagcttaattgaagaatcgcagaaccaacaagaaaagaatgaactagacttattacaat
tggataagtgggcaagttgtggaattggttgacataacataag

FIG.5

Docket No. #3
1038-1176 MIS:jb

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

EXPRESSING GP140 FRAGMENT OF PRIMARY HIV-1 ISOLATE

the specification of which
(check one)

☐ is attached hereto.

☒ was filed on **February 24, 2000** as United States Application No. or PCT International Application Number **PCT/CA00/00190**

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

09/256,194	**February 24, 1999**	**Pending**
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
PCT/CA00/00190	**February 24, 2000**	
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

1 **Michael I. Stewart (Reg. No. 24,973)**

Send Correspondence to: **Sim & McBurney**
6th Floor, 330 University Avenue
Toronto, Ontario
Canada, M5G 1R7.

Direct Telephone Calls to: *(name and telephone number)*
(416) 595-1155

Full name of sole or first inventor **Charles D.Y. Sia**
Sole or first inventor's signature Date
Residence **Thornhill, Ontario Canada** CAX
Citizenship **Canadian**
Post Office Address **189 Mabley Crescent, Thornhill, Ontario, Canada, L4J 2Z7.**

Full name of second inventor, if any **Shi-Xian Cao**
Second inventor's signature Date Sept. 24, 2001
Residence **Stouffville, Ontario, Canada** CAX
Citizenship **Canadian**
Post Office Address **12455 Ninth Line, #204, Stouffville, Ontario, Canada, L4A 1J3**

300

Full name of third inventor, if any
Roy Persson

Third inventor's signature Roy Persson Date September 24, 2001

Residence
Toronto, Ontario, Canada CAN

Citizenship
Canadian

Post Office Address
7 Bishop Avenue, Unit 604, Toronto, Ontario, Canada, M2M 4J4.

Full name of fourth inventor, if any
Benjamin Rovinski

Fourth inventor's signature Date

Residence
Thornhill, Ontario, Canada

Citizenship
Canadian

Post Office Address
70 Winding Lane, Thornhill, Ontario, Canada, L4J 5H6

500

Full name of fifth inventor, if any
Mark Parrington

Fifth inventor's signature Mark Parrington Date Oct. 12, 2001

Residence
Bradford, Ontario, Canada CAN

Citizenship
Canadian

Post Office Address
45 Martin Street, Bradford, Ontario, Canada, L3Z 1Z4.

Full name of sixth inventor, if any

Sixth inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of third inventor, if any
Roy Persson

Third inventor's signature Roy Persson Date September 24, 2001

Residence
Toronto, Ontario, Canada

Citizenship
Canadian

Post Office Address
7 Bishop Avenue, Unit 604, Toronto, Ontario, Canada, M2M 4J4.

400

Full name of fourth inventor, if any
Benjamin Rovinski

Fourth inventor's signature Benjamin Rovinski Date November 5 2001

Residence
Thornhill, Ontario, Canada CAN

Citizenship
Canadian

Post Office Address
BR BR
~~**70 Winding Lane**~~, **Thornhill, Ontario, Canada,** ~~**L4J 5H6**~~ L3T 5N2
40 LUNAU LANE

Full name of fifth inventor, if any
Mark Parrington

Fifth inventor's signature Mark Parrington Date Oct. 12, 2001

Residence
Bradford, Ontario, Canada

Citizenship
Canadian

Post Office Address
45 Martin Street, Bradford, Ontario, Canada, L3Z 1Z4.

Full name of sixth inventor, if any

Sixth inventor's signature Date

Residence

Citizenship

Post Office Address